

REMARKS/ARGUMENTS

The Advisory Action of April 7, 2009, has been received and carefully reviewed. The Examiner states that the previous Reply, dated March 31, 2009, was not entered because the Applicant was arguing limitations not claimed and discussing new evidence that was not considered. Without acquiescing to the Examiner's objection, Applicant now submits this Reply along with a Request for Continued Examination, and the required fees. Entry of the Reply is requested.

Status of the Claims

Claims 1, 3, 4 and 7 have been amended. Claims 2 and 5-6 are canceled. Claims 1, 3, 4, and 7 are pending. Claims 8-47 are withdrawn.

Applicant has amended claims 1, 3, 4 and 7 to more clearly define that which Applicant considers to be the invention. Specifically, Applicant has included language to further distinguish Applicant's claimed invention, from the VG4 antibody of Arystarkhova et al. cited previously. Applicant's claim now states that the claimed antibody was made to the synthetic purified amino acid sequence of SEQ ID NO: 1. Support for this amendment can be found throughout the specification, including, but not limited to, page 38, lines 1-10.

Furthermore, Applicant has also amended claim 1 to indicate that the binding of the claimed antibody also increases (Na⁺+K⁺)-ATPase activity and cardiac contractility. Support for these amendments can be found in the specification at pages 39-40, 41-43, and Kai Xu, "Dual Activity of the H1-H2 domain of the (Na⁺+K⁺)-ATPase", *Biochem. Biophys. Res. Comm.* 377 (2008) 469-473 (Attached as Appendix A). In addition, Applicant has also amended claim 1 to include the feature that the antibody be administered in an amount sufficient to provide the claimed effect. Support for this amendment can be found in Example 1, pages 42-43 of Applicant's specification.

Applicant amended claim 3 to delete the phrase "prevention of heart disease", and amended claim 3 and 7 to include the phrase "or heart muscle contractile disorders". Support for these amendments can be found, *inter alia*, in Applicant's specification at page 3, lines 20-27 and page 4, lines 26-29. In addition, Applicant amended claim 4 to clarify that the

composition of claim 1 could be a component of a vaccine. Support for this amendment can be found in Applicant's specification at pages 34-37. No new matter has been added by these amendments.

Discussion of the New Matter Rejection

The Examiner rejected claims 1, 3, 4 and 7 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. According to the Examiner, the specification does not appear to provide blazemarks nor direction for the term "cardiac isoforms" included in the claims. Applicant respectfully traverses this rejection.

To begin, the Applicant amended claim 1 by deleting the term "cardiac" as superfluous in view of the context of the claim. The term "isoform" is understood by those of ordinary skill to mean that the specific amino acid sequence that is bound by the claimed antibody can vary by one or more amino acids, depending on alternative gene splicing, single nucleotide polymorphisms, etc. The antibody of the present invention is specifically directed to isoforms of the amino acid sequence of SEQ ID NO: 1, which is found in the H1-H2 domain of the α subunit of the $(\text{Na}^+ + \text{K}^+)$ -ATPase in heart tissue (page 21 of the specification). Therefore, the term "and isoforms thereof" is meant to define the scope of the isoforms claimed, as those known isoforms of the amino acid sequence of the H1-H2 domain associated with the α subunit of the $(\text{Na}^+ + \text{K}^+)$ -ATPase enzyme found in the myocytes of heart tissue of various vertebrate species, at the time the application was filed. For example, on page 13698 of Arystarkhova et al., isoforms of the α subunit of the $(\text{Na}^+ + \text{K}^+)$ -ATPase from different species and different organs are shown. Moreover, it is known that rat has two isoforms in the heart (see, P.A. Lucchesi, et al., "Postnatal changes in $(\text{Na}^+ + \text{K}^+)$ -ATPase isoform expression in rat cardiac ventricle: Conservation of biphasic ouabain affinity", *J. Biol.Chem.* 266 (1991) 9327-9331; A.A. McDonough, et al., "Subcellular distribution of sodium pump isoform subunits in mammalian cardiac myocytes", *Am. J. Physiol.* 270 (1996) C1221-1227), and there are three known isoforms of the α subunit of the $(\text{Na}^+ + \text{K}^+)$ -ATPase in the human heart (see also, A.A. McDonough, et al., "The cardiac sodium pump: structure and function", *Basic Res. Cardiol.* 97 (Suppl. 1) (2002) I19-24; J. Wang, et al., "All human $\text{Na}(+) - \text{K}(+) - \text{ATPase}$ alpha-subunit isoforms have a similar affinity for cardiac glycosides", *Am. J. Physiol. Cell Physiol.* 281 (2001) C1336-1343.

Applicant submits with this Reply, a recently published paper in which Applicant's own experimental results show that both the monoclonal and polyclonal antibodies of the present invention specifically bind, and exert positive (Na⁺+K⁺)-ATPase activation, in mice, dog and rat isoforms of the enzyme (see, for example, Applicant's specification, Examples, pages 37-42, and Kai Xu, at page 471, and FIGS 2 and 4 (Appendix A). Thus it is clear to one of ordinary skill in the art, that scope of the antibodies claimed by Applicant are defined as those antibodies that are made against the peptide of SEQ ID NO: 1, and those variants which are derived from the isoforms of the H1-H2 domain of the α subunit of the (Na⁺+K⁺)-ATPase found in the cardiac myocytes of vertebrates, and more specifically, found in the cardiac myocytes of mammals, such as humans, dogs, mice, rats, pigs, etc. As such, the claim term "and isoforms thereof" is sufficiently described and supported by the specification, as well as the data published subsequently in Appendix A, and in the prior art. As such, Applicant respectfully requests withdrawal of the rejection.

Discussion of the Written Description Rejections

The Examiner rejected claims 1, 3, 4 and 7 under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. According to the Examiner, the phrase "and cardiac isoforms thereof" in claim 1, lacks sufficient antecedent basis in that it is unclear if this refers to "cardiac isoforms of the sequence RSATEEEPPNDD" or "cardiac isoforms of the α -subunit of (Na⁺+K⁺)-ATPase enzyme. Applicant respectfully traverses this rejection.

In response to the rejection, Applicant submits that the antibodies claimed by Applicant are defined as those that are generated to the peptide of SEQ ID NO: 1, and those variants derived from the isoforms of the H1-H2 domain of the α subunit of the (Na⁺+K⁺)-ATPase found in the cardiac myocytes of vertebrates, and more specifically, found in the cardiac myocytes of mammals, such as humans, dogs, mice, rats, pigs, etc. The discussion in Applicant's specification regarding the number of possible amino acid variations is provided so that one of ordinary skill would understand that there could be more than one substitution of an amino acid for another in SEQ ID NO: 1, yet the antibody can still bind and exert its therapeutic effect. The isoform of a protein represents any different amino acid sequence of the same protein or peptide formed due to single nucleotide polymorphisms or alternative gene splicing. As stated above, at least three isoforms of the (Na⁺+K⁺)-ATPase, including

$\alpha 1$, $\alpha 2$, and $\alpha 3$, have been found in human heart (See Kai Xu, Appendix A, page 469). These isoforms of the ($\text{Na}^+ + \text{K}^+$)-ATPase share the same enzymatic function, even though there are specific differences in the primary structure of the enzyme. Thus, one of ordinary skill in the art would understand that Applicant's claim would cover those variants as long as they met the limitations of specific binding and exerting its claimed effect on heart tissue. Applicant respectfully requests withdrawal of this rejection.

The Examiner also rejected claims 1, 3 and 4 under 35 U.S.C. 112, first paragraph, because the Examiner alleges that the specification, while being enabling for the recited composition for treatment of heart failure, did not provide enablement for the term "prevention of heart failure". Applicant submits that one of ordinary skill in the art would understand that prevention of further heart failure would be expected upon treatment of a patient with the claimed invention. However, in the interest of furthering prosecution of the instant application, and in no way conceding to the Examiner's view, Applicant has amended the claim 3 to delete the term "prevention". Applicant requests withdrawal of this rejection as moot.

Discussion of the Novelty Rejection

The Examiner again rejected claims 1, 3, 4, and 7 under 35 U.S.C. §102(b) as anticipated by Arystarkhova et al., as evidenced by Bost et al. (Immunol. Invest. 1988; 17:577-586), Bendayan et al. (J. Histochem. Cytochem. 1995; 43:881-886) and the instant specification at page 3, 3rd paragraph and page 43-44. The rejection is essentially identical to that made in the previous Office Action of record. The Examiner contends that Applicant's claims do not recite any particular structure for the claimed antibody, thus, the claimed antibody is defined by its binding specificity and its ability to increase myocyte intracellular diastolic and systolic calcium upon binding to the amino acid sequence. Applicant respectfully traverses this rejection.

Applicant has now amended claim 1 to recite that the claimed antibody is made against the synthetic peptide of SEQ ID NO: 1, which further distinguishes Applicant's claimed Jianye-2 antibody from the VG4 antibody of Arystarkhova et al., because VG4 was made by immunization of rabbits with the entire ($\text{Na}^+ + \text{K}^+$)-ATPase enzyme obtained from pig kidney. One of ordinary skill in the immunology arts would understand that antibodies made using these two different methods, the purified peptide of 12 amino acids, versus an

entire protein of hundreds of amino acids, could not be identical in structure or function. While there may be cross reactivity to some extent depending on epitopes available in the whole protein, they could not be identical.

The Examiner contends that the VG4 antibody of Arystarkhova et al. binds an epitope composed primarily of contiguous amino acids QAATEEEPQNDNL of pig $\alpha 1$ ($\text{Na}^+ + \text{K}^+$)-ATPase, and because Arystarkhova teaches an antibody that binds to the pig $\alpha 1$ ($\text{Na}^+ + \text{K}^+$)-ATPase H1-H2 loop and "inhibits enzyme activity up to 50%", the properties of the antibody of Arystarkhova et al. are consistent with the antibody increasing "positive inotropic activity in cardiac tissue". Therefore, according to the Examiner, if the antibodies of Arystarkhova et al. have the same binding affinity and same function, then they anticipate Applicant's claimed composition. However, as Applicant will show, this is not the case.

Applicant's claimed Jianye-2 antibodies surprisingly, and unexpectedly, have opposite enzymatic effects than the effects of the VG4 antibody taught in Arystarkhova et al., and therefore cannot anticipate or render obvious, Applicant's claimed invention, as explained below.

First, Arystarkhova et al. teach that the VG4 antibody inhibits ($\text{Na}^+ + \text{K}^+$)-ATPase enzyme activity (ATP hydrolysis, (Fig 9)). The enzyme is inhibited up to 50% when compared to control IgG (Arystarkhova, page 13699). In contrast, Applicant's purified monoclonal and polyclonal antibodies made to SEQ ID NO: 1 according to the specification, specifically bind that portion of the H1-H2 domain of the α subunit of the ($\text{Na}^+ + \text{K}^+$)-ATPase and increase the activity of ($\text{Na}^+ + \text{K}^+$)-ATPase. Applicant invites the Examiner to review the published data by the Applicant in Appendix A, which uses the method detailed in Applicant's specification at pages 41-42. Specifically at page 471 of Appendix A, the results demonstrate that in rat and dog isoforms of ($\text{Na}^+ + \text{K}^+$)-ATPase, the binding of the antibodies of Applicant's claimed invention to the α subunit of the ($\text{Na}^+ + \text{K}^+$)-ATPase resulted in up to a 180% increase in enzyme activity over control (Fig. 2, Appendix A).

Second, Arystarkhova teaches that VG4 antibodies enhance ouabain mediated inhibition of ATP hydrolysis (page 13699, Fig. 10). In contrast, Applicant's Jianye-2 antibodies inhibit or block ouabain's effect on ($\text{Na}^+ + \text{K}^+$)-ATPase in rat and dog isoforms (Fig. 3, Appendix A).

Third, as the Examiner stated in the Office Action, it was thought that inhibition of the $(\text{Na}^+ + \text{K}^+)$ -ATPase was what caused positive inotropic effects in heart muscle. However, Applicant has now shown that this is surprisingly not the case. Applicant's claimed antibodies actually increase $(\text{Na}^+ + \text{K}^+)$ -ATPase activity and show a positive inotropic effect in rats. See, for example, Applicant's specification at Example 1, figures 2-6 and Appendix A, page 472 and Fig. 4. These findings are contrary to what anyone of ordinary skill in the art would have predicted for an antibody binding to the H1-H2 region of the α subunit of the $(\text{Na}^+ + \text{K}^+)$ -ATPase, as claimed by Applicant.

Applicant summarizes the differences between VG4 and Applicant's claimed antibody in Table I below.

TABLE I

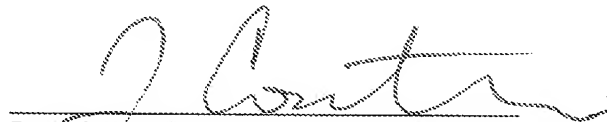
Antibody Name	Fundamental Distinction in How to Make Original Antigen	Fundamental Distinction in Antibody Specificity	Fundamental Distinction in Antibody Biological Function	Fundamental Distinction in How to Use Antibody
VG4	Use entire (Na ⁺ +K ⁺)-ATPase including α and β subunits isolated from pig kidney to generate original VG4 antibody.	VG4 does not recognize denatured (Na ⁺ +K ⁺)-ATPase and does not bind to purified RSATEEEPPNDD peptide and denatured enzyme.	VG4 increases ouabain binding and Inhibits the (Na ⁺ +K ⁺)-ATPase activity.	For assumed three dimensional Enzyme structure experiments.
Jianye-2	Use specific purified synthetic RSATEEEPPNDD peptide, SEQ ID NO: 1 to generate original Jianye-2 antibody	Jianye-2 specifically binds to RSATEEEPPNDD site for both native and denatured (Na ⁺ +K ⁺)-ATPase.	Jianye-2 antibody decreases ouabain binding and activates the (Na ⁺ +K ⁺)-ATPase, which in turn, increases intracellular Ca and cardiac contraction.	Use isolated Jianye-2 for passive immunization and use antigen of Jianye-2 as vaccine to generate endogenous Jianye-2 for active immunization. Both immunotherapies can be used for new treatments of heart failure.

Applicant submits that the prior art, taken as a whole, in the proper context, and in view of the latest scientific results, does not provide a reasonable scientific basis for the Examiner continue to assert that the VG4 antibody has the same structure and function as

Applicant's claimed invention. Applicant has demonstrated that the Applicant's claimed invention cannot be anticipated by the VG4 antibodies of Arystarkhova, because even though the antibodies of both Arystarkhova and Applicant may have cross reactivity, the antibodies were made using different methods, and they have completely different functional attributes. Therefore, Applicant's claimed invention cannot be assumed to be inherently taught in the prior art antibodies of Arystarkhova. As such, Applicant respectfully requests withdrawal of the rejection.

Applicants respectfully submit that the patent application is in condition for allowance. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



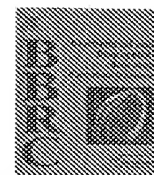
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APPENDIX A



Dual activity of the H1-H2 domain of the (Na⁺+K⁺)-ATPase

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ABSTRACT

(Na⁺+K⁺)-ATPase is a target receptor of digitalis (cardiac glycoside) drugs. It has been demonstrated that the H1-H2 domain of the α -subunit of the (Na⁺+K⁺)-ATPase is one of the digitalis drug interaction sites of the enzyme. Despite the extensive studies of the inhibitory effect of digitalis on the (Na⁺+K⁺)-ATPase, the functional property of the H1-H2 domain of the enzyme and its role in regulating enzyme activity is not completely understood. Here we report a surprise finding: instead of inhibiting the enzyme, binding of a specific monoclonal antibody SSA78 to the H1-H2 domain of the (Na⁺+K⁺)-ATPase elevates the catalytic activity of the enzyme. In the presence of low concentration of ouabain, monoclonal antibody SSA78 significantly protects enzyme function against ouabain-induced inhibition. However, higher concentration of ouabain completely inactivates the (Na⁺+K⁺)-ATPase even in the presence of SSA78. These results suggest that the H1-H2 domain of the (Na⁺+K⁺)-ATPase is capable of regulating enzyme function in two distinct ways for both ouabain-sensitive and -resistant forms of the enzyme: it increases the activity of the (Na⁺+K⁺)-ATPase during its interaction with an activator; it also participates in the mechanism of digitalis or ouabain-induced inhibition of the enzyme. Understanding the dual activity of the H1-H2 domain will help better understand the structure-function relationships of the (Na⁺+K⁺)-ATPase and the biological processes mediated by the enzyme.

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Sodium- and potassium-dependent triphosphatase [(Na⁺+K⁺)-ATPase or NKA] [1] is a key membrane protein that couples the hydrolysis of ATP to the opposite vectorial transport of Na⁺ and K⁺ ions across the plasma membrane, as needed for the primary source of energy for the active transport of various nutrients, electrical excitability, cellular ion homeostasis, uptake of neurotransmitters, and regulation of cell volume that are vital to the cell living processes [2–5]. NKA consists of α - and β -subunits [6,7]. The catalytic α -subunit contains the sites for binding of Na⁺, K⁺, and ATP [6–9]. The β -subunit appears to be essentially required for biosynthesis, maturation, and migration of the α -subunit to the plasma membrane [10,11]. The crystal structure of NKA suggests that the α -subunit traverses the membrane ten times and both the N- and C-terminals are located on the cytoplasm side [12]. The β -subunit contains only one hydrophobic region, and only the N-terminal is located on the cytoplasm side [12]. Several isoforms of α - and β -subunits have been identified [13–16]. There are two α isoforms ($\alpha 1$ and $\alpha 2$) of NKA in rodent heart [17,18] and three α isoforms ($\alpha 1$, $\alpha 2$, and $\alpha 3$) in human heart [19,20].

One of the striking properties of NKA is its ability to regulate cardiac contractility [21,22]. NKA has been a target receptor for digitalis and related cardiac glycosides drugs for the treatment of heart failure for more than 200 years [23]. The digitalis drug-induced positive inotropic effect is dependent on the inhibition of NKA catalytic activity and impairment of active transport of Na⁺/

K⁺ ions in heart cells [24]. Studies have demonstrated that the extracellular H1-H2 domain of the α -subunit of NKA participates in the ouabain binding to the enzyme and that the border positions of the H1-H2 domain are essential to the ouabain sensitivity of the enzyme [25,26]. However, our previous studies have shown that the H1-H2 domain of the α -subunit of NKA is a critical determinant of the biological activity of the enzyme, which couples to enhanced myocyte calcium transient and inotropic action but without inhibition of NKA [27]. This discrepancy draws our attention to the native activity of the H1-H2 domain. To date, it remains obscure whether any molecular interactions at the H1-H2 domain would cause inhibition of NKA function. The nature of the H1-H2 domain in regulating NKA function and in the mechanism of the digitalis-induced inhibition has not been completely demonstrated.

The strategy to explore the native activity of the H1-H2 domain of NKA is to use specific monoclonal antibody SSA78 (mAb SSA78) and to monitor the changes of NKA activity during the antibody-protein interaction at the H1-H2 domain site with or without ouabain. The experimental results reported here reveal a hidden activity of the H1-H2 domain of the α -subunit of NKA that may shed new light to aid understanding of the molecular mechanism underlying NKA-mediated biological processes.

Materials and methods

Materials. Ouabain (>99%) was purchased from Fluka BioChemika (Buchs, Switzerland). Other reagents were from Sigma Chemical.

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Purified mAb SSA78 and polyclonal antibody SSA78 (pSSA78) were generated against the H1-H2 domain (¹¹⁸RSATEEEPPNDD¹²⁹) of the $\alpha 1$ -subunit of rat NKA (Cytomol, CA, USA). Synthetic peptide RSATEEEPPNDD (BioSynthesis, TX, USA) was used as a peptide blocker (PB78) in the study. Frozen dog heart muscle was a gift of Jack Kyte's laboratory. Sprague Dawley rats and spontaneous hypertension heart failure (SHHF) rats were purchased from Charles River Laboratories (Wilmington, MA, USA). The Animal Care and Use Committees of the University of Maryland School of Medicine approved the animal protocols.

Purification of cardiac NKA. NKA was purified from rat and dog heart muscle separately using an independent protocol as described previously [28]. (A) Preparation of sarcolemmal vesicles: Briefly, animal hearts were rinsed in an ice-cold buffer solution containing 10 mM histidine (free base) and 0.75 M NaCl. The left ventricles were cut into 5 mm pieces and homogenized for 10 s at 15,000 rpm. The homogenates were centrifuged at 10,000 rpm for 20 min. The supernatant was discarded, and the pellets were resuspended in 10 mM NaHCO₃ and 5 mM histidine. The pellets were resuspended, homogenized, and centrifuged for two more times as described above to remove the sarcoplasmic reticulum (SR) for the ultimate isolation of sarcolemmal vesicles (SL). The pellets obtained from the third centrifugation described above were resuspended in 10 mM NaHCO₃ and 5 mM histidine and homogenized three times for 30 s at 15,000 rpm. The pellets were then sedimented at 10,000 rpm for 20 min, and the supernatant containing the isolated SL vesicles was centrifuged again for 30 min at 20,000 rpm and the supernatant was discarded. The pellets resulting from this centrifugation were resuspended in 1.0 M sucrose, 0.3 M NaCl, 50 mM tetrasodium pyrophosphate, and 0.1 M Tris (pH 7.1), and loaded on the bottom of the centrifuge tubes. A solution (containing 0.6 M sucrose, 0.3 M NaCl, 50 mM tetrasodium pyrophosphate, and 0.1 M Tris, pH 7.1) was next layered on the top of the membrane suspension in each tube. The solution (containing 0.25 M sucrose and 10 mM histidine) was then layered on top of the 0.6 M sucrose in each tube. Samples were centrifuged for 60 min at 60,000 rpm. After the run, the SL vesicles were observed

as distinct snow-white protein bands at the interfaces of the 0.25 M/0.6 M sucrose layers. The SL vesicles were sedimented at 40,000 rpm for 40 min. The final SL vesicle suspension was collected for further purification. (B) Preparation of SDS-treated cardiac NKA: Rat SL vesicles (4.4 mg/ml) were titrated with 0.58 mg/ml of SDS in the presence of 2 mM ATP at 20 °C for 30 min and then loaded on the top of a sucrose (W/W) step gradient (15%, 28.8%, and 37.3%) in a Ti 60 tube and centrifuged at 40,000 rpm for 90 min. The fractions containing NKA were collected and stored at -70 °C. The specific enzymic activity of NKA in these preparations was 600, 1000, and 450 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ for rat, dog, and SHHF rat NKA, respectively.

Measurement of NKA activity. NKA activity was determined on the basis of Jack Kyte's method with modifications as previously described [29] under various experimental conditions. The enzymatic activity is defined as the ouabain-sensitive hydrolysis of MgATP in the presence of Na⁺ and K⁺. NKA activity is defined as ouabain-sensitive enzyme activity in different experiments. Purified ouabain-resistant rat NKA and ouabain-sensitive dog NKA were incubated with or without different concentrations of mAb SSA78 at 4 °C for 60 min. The reaction was initiated by adding MgATP (3 mM) in a final volume of 0.2 ml at 37 °C for 30 min and terminated by adding 0.75 ml quench solution (0.5% ammonium molybdate + 0.5 M H₂SO₄) and 0.02 ml developer (25 mg/ml of the mixture of 0.2 g 1-amino-2-naphthol-4-sulfonic acid + 1.2 g sodium bisulfate + 1.2 g sodium sulfite). Color was developed for 30 min at room temperature and the concentration of phosphate was then determined at 700 nm using a spectrophotometer. In the purified enzyme system, incubation of NKA and specific antibody for 60 min prior to initiate ATPase assay is an optimal condition to obtain a significant activation of the enzyme.

Immunofluorescent staining. Rat myocytes were frozen and cut on a cryostat. Sections (8 μm) of each tissue were blocked with 1% bovine serum albumin (BSA) and incubated with mAb SSA78 (1:1000) for 60 min in the presence or absence of 5 mM ouabain or PB78. Washed slides were evaluated after incubation with a FITC conjugated goat anti-rabbit antibody (1:75) as described previously [28].

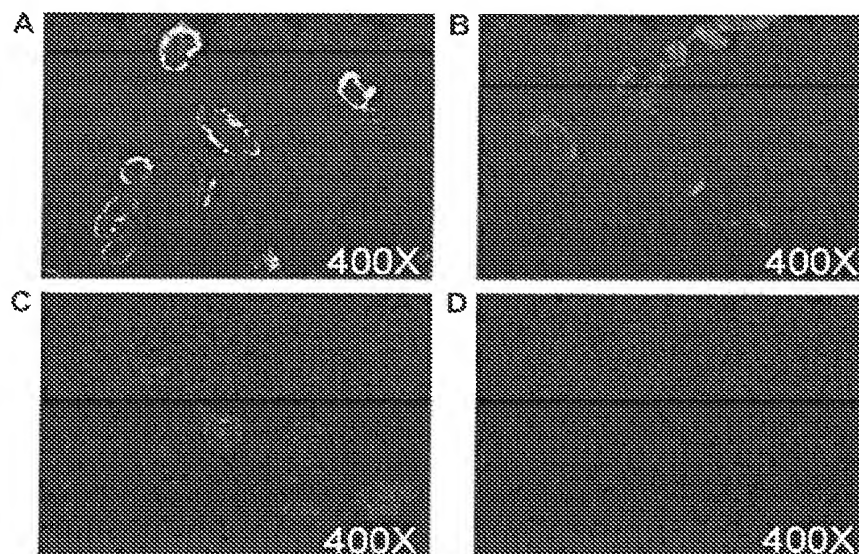


Fig. 1. Immunofluorescent staining of mAb SSA78 on rat myocytes. Isolated rat cardiac myocytes were frozen and cut on a cryostat. Sections (8 μm) of each tissue were blocked with 1% BSA and incubated with mAb SSA78 (1:1000) for 60 min at room temperature with or without 5 mM ouabain or PB78. All confocal images were at a magnification of 400 \times . (A) a group of cells with mAb SSA78, (B) with ouabain in condition A, (C) with PB78 in condition A, and (D) secondary antibody control. The results show that PB78 eliminates and ouabain competes with mAb SSA78 binding to the H1-H2 domain of NKA. Each of the data represents one of three similar stainings.

Results

The H1-H2 domain is the specific antigenic site of mAb SSA78

We first examined the specificity of mAb SSA78 by immunofluorescent staining since it is an important tool for the study. The results show that mAb SSA78 specifically labeled the H1-H2 domain of NKA on the cell membrane surface (Fig. 1A). In contrast, no labeling occurred in the absence of mAb SSA78 (Fig. 1D). Ouabain competed with the binding of mAb SSA78 and strongly reduced the immunofluorescent staining (Fig. 1B). Peptide blocker PB78 had exactly the same composition as of the H1-H2 domain that can saturate the functional sites of mAb SSA78 and completely eliminated the capability of mAb SSA78 to bind to its specific antigenic site on NKA (Fig. 1C).

Binding of mAb SSA78 to the H1-H2 domain increased NKA activity

Having established the specificity of mAb SSA78 that binds to the H1-H2 domain and competes with ouabain binding to NKA, we next investigated whether interaction of mAb SSA78 at the H1-H2 domain would cause inhibition of NKA activity like ouabain does. Experimental results reveal that NKA activity is a function of the concentration of mAb SSA78 (Fig. 2). Binding of mAb SSA78 to either ouabain-resistant rat NKA or ouabain-sensitive dog NKA increased the catalytic activity of the enzyme (Fig. 2). The activity of ouabain-resistant rat NKA was 137 ± 16 , 148 ± 21 , 154 ± 19 , 162 ± 14 , 167 ± 16 , 174 ± 13 , and $182 \pm 10\%$ in the presence of 0.1, 0.2, 0.3, 0.5,

0.7, 1.0, and 2.0 mM mAb SSA78 compared with the control (in the absence of mAb SSA78) as shown in Fig. 2A. Under the same experimental condition for the concentration of mAb SSA78, the activity of ouabain-sensitive dog NKA was 133 ± 4.0 , 142 ± 11 , 149 ± 6.0 , 157 ± 5.0 , 165 ± 9.0 , 174 ± 12 , and $176 \pm 17\%$ (Fig. 2B). The half effective concentrations (EC_{50}) for rat NKA and dog NKA were 0.141 and 0.154 μ M, respectively (Fig. 2).

Protective effect of mAb SSA78 on NKA activity in ouabain-induced inhibition

We next tested the changes of NKA activity when both ouabain and mAb SSA78 interacted at the H1-H2 domain of the enzyme. In the presence of 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , and 10^{-3} M ouabain without mAb SSA78, rat NKA activity was reduced to 95 ± 10 , 96 ± 8.0 , 89 ± 7.0 , 83 ± 14 , 74 ± 9.0 , 46 ± 10 , and $11 \pm 3.0\%$ (Fig. 3A) compared with the control without ouabain, and 100 ± 6.0 , 84 ± 27 , 60 ± 22 , 25 ± 11 , 10 ± 6.0 , 3 ± 2.0 , and $1.3 \pm 1.0\%$ for dog NKA (Fig. 3B). Significant changes of rat and dog NKA activities were detected in the presence of 1 μ M mAb SSA78 with the same ouabain concentration as indicated above: ouabain-resistant rat NKA activity was 164 ± 14 , 155 ± 17 , 150 ± 23 , 140 ± 22 , 124 ± 2.0 , 68 ± 3.0 , and $12 \pm 1.0\%$ (Fig. 3A) compared with the control (without ouabain and mAb SSA78), and 162 ± 22 , 146 ± 18 , 126 ± 35 , 75 ± 33 , 5.3 ± 2.0 , 0 ± 0 , and $0 \pm 0\%$ for ouabain-sensitive dog NKA (Fig. 3B). mAb SSA78 partially protected rat and dog NKA activity, but not in the case of high concentration of ouabain (Fig. 3A and B).

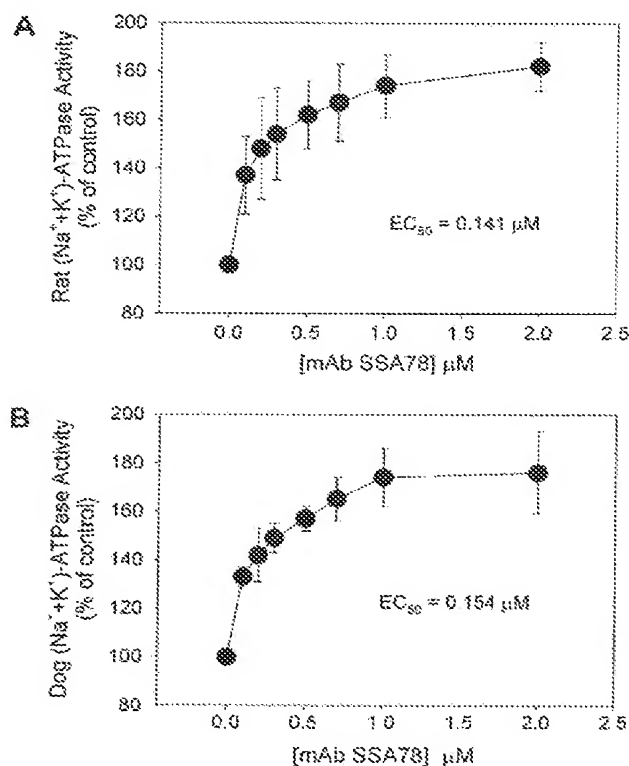


Fig. 2. Effect of mAb SSA78 on the catalytic activity of NKA. Purified rat NKA (7.5 μ g/ml) and dog NKA (1.3 μ g/ml) were incubated with different concentrations of mAb SSA78 (as indicated in the figure) for 60 min at 4 °C in the presence of 100 mM Na⁺ and 20 mM K⁺ prior to ATPase assay. NKA activity significantly increased in the presence of mAb SSA78. Each data point represents the mean of four independent experiments.

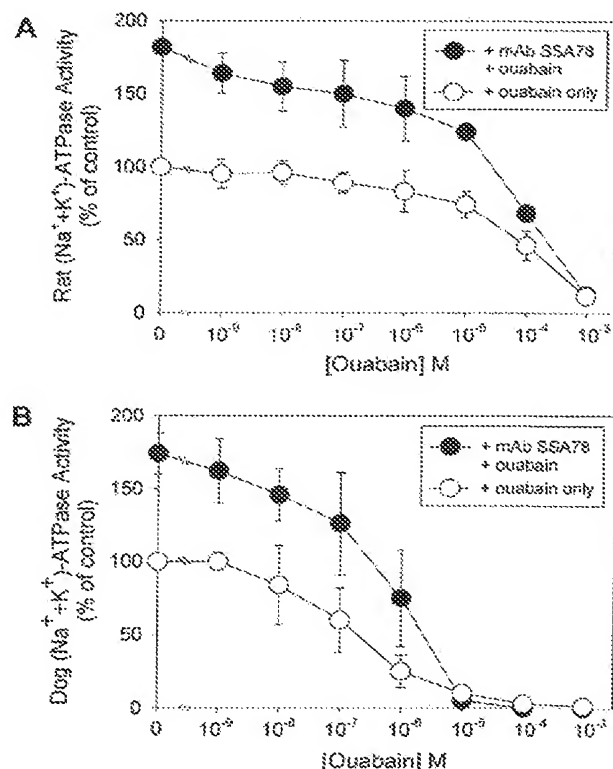


Fig. 3. Effect of mAb SSA78 on the ouabain-induced inhibition of NKA. Purified rat NKA (7.5 μ g/ml) and dog NKA (1.3 μ g/ml) were incubated with (black circles) or without (open circles) 1 μ M mAb SSA78 in the presence of different concentrations of ouabain as indicated in the figure for 60 min at 4 °C prior to the enzyme activity assay. At low concentration of ouabain, SSA78 markedly elevates and protects NKA activity against ouabain-induced inhibition; higher concentration of ouabain completely inhibits NKA activity. Each data point represents the mean of six independent experiments.

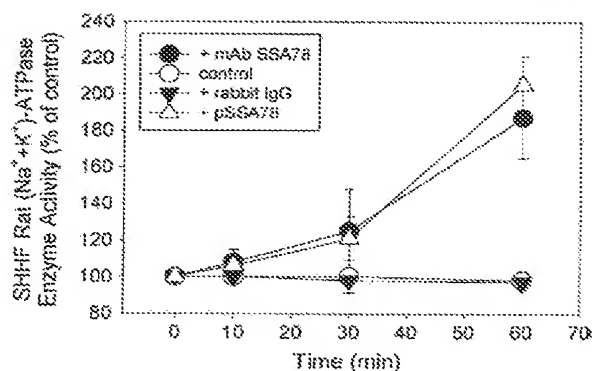


Fig. 4. Time course of the effect of mAb SSA78 and pSSA78 on SHHF NKA isolated from heart failure rat. Cardiac SHHF NKA (7.5 µg/ml) was incubated with or without mAb SSA78, or pSSA78, or total rabbit IgG (1 µM each) in different time course as indicated in the figure. Open circles, control; black circles, with mAb SSA78; open triangle, with pSSA78; black triangle, with rabbit IgG. SHHF NKA activity was significantly increased in the presence of mAb SSA78 or pSSA78 after 60 min incubation. No changes of enzyme function were detected in the absence of both mAb SSA78 and pSSA78, or total rabbit IgG. Each data point represents the mean of three independent experiments.

Both mAb SSA78 and pSSA78 increase enzyme activity by interaction at the H1-H2 domain of NKA isolated from SHHF rat

We further tested the effect of mAb SSA78 and pSSA78 [27] on purified cardiac NKA that was isolated from heart failure rat model by interacting on the H1-H2 domain of the enzyme. By comparing with the control sample (without antibody), Fig. 4 shows that by comparing with the control sample (without antibody), purified SHHF NKA activity was increased to 108 ± 7.0 , 125 ± 23 , and $187 \pm 22\%$ in the presence of 1 µM mAb SSA78 (Fig. 4, black circles), and 106 ± 4.0 , 121 ± 12 , and $205 \pm 16\%$ with 1 µM pSSA78 (Fig. 4, open triangles) during 10, 30, and 60 min interaction time course at the H1-H2 domain of the enzyme. No significant changes were observed in the control sample (Fig. 4, open circles) and the samples in the presence of rabbit IgG (black triangles). No inhibition of SHHF NKA was detected in the presence of mAb SSA78 or pSSA78 (Fig. 4).

Discussion

A hidden activity resides within the H1-H2 domain of NKA

To gain a detailed understanding of the functional activity of the H1-H2 domain of NKA, we prepared a simple system consisting of a highly purified NKA in a medium containing only small ions, buffer molecules, and specific antibody that made against the H1-H2 domain of the enzyme. Under the experimental conditions described in the methods, we discovered that specific antibody-protein interaction at the H1-H2 domain of the α -subunit of NKA accelerates the catalytic activity of the enzyme (Figs. 2 and 4). This is the first time that this hidden property of the H1-H2 domain of NKA has been reported.

Dual activity of the H1-H2 domain

It has been well demonstrated that the Gln-111 and Asn-122 of the H1-H2 domain interact with ouabain and directly regulate the affinity of ouabain binding to NKA [25,26]. Our data clearly show that mAb SSA78 (1 µM) significantly protects both ouabain-resistant and -sensitive NKA activity against ouabain-induced inhibition (Fig. 3A and B), indicating that the H1-H2 domain of NKA participates in

both activation and inhibition processes mediated by the enzyme. NKA activity was markedly elevated by the interaction of mAb SSA78 at the H1-H2 domain of the enzyme (Figs. 2 and 4), suggesting that the H1-H2 domain is capable of regulating NKA function by enhancing the catalytic activity of the enzyme when it interacts with an activator, such as mAb SSA78 or pSSA78 (Fig. 4). The fact that ouabain competes with specific mAb SSA78 binding to NKA (Fig. 3) provides further evidence to support the notion that ouabain binds to the H1-H2 domain and is involved in the mechanism of the ouabain-induced inhibition of the enzyme [30].

It is evident that inhibitor ouabain and activator mAb SSA78 (or pSSA78) affect NKA biological function in a dramatically opposite direction through the interaction at the H1-H2 domain (Figs. 2–4). However, neither inhibitor nor activator would change the basic nature of NKA function or create new functions for the enzyme. This fundamental limitation of both inhibitor and activator of NKA suggests that the primary structure of the H1-H2 domain of the enzyme is responsible for its dual activity by dictating different drug actions to influence NKA activity, presumably by controlling ligand-induced conformational changes in the enzyme.

Extensive investigations have demonstrated that in addition to the H1-H2 domain, the H3-H4 and H5-H6 hairpins of NKA also bind ouabain with high affinity and that NKA inhibition is digitalis-mediated paralysis of H5-H6 domain [31,32]. Our experimental results reveal that NKA activity was completely destroyed at high concentrations of ouabain (1 mM for ouabain-resistant rat NKA and 10 µM for ouabain-sensitive dog NKA) in the presence of mAb SSA78 as shown in Fig. 3. The fact that mAb SSA78 failed to enhance or maintain NKA activity in the presence of high concentration of ouabain suggests that other amino acids residing within the H3-H4 and H5-H6 hairpins may play significant roles in the inhibition of NKA. High concentration of ouabain may cause radical shifts in the balance of forces between side chains of NKA, leading to different conformation of the enzyme to favor ouabain inhibition. Several laboratories have also reported that low concentration of ouabain stimulates NKA activity under different experimental conditions [33]. This may be explained by the possibility that low concentration of ouabain might randomly contact the H1-H2 domain first before interacting with other domains of NKA during its binding process, and thus elevated enzyme activity may be observed since the H1-H2 domain has a latent ability to influence NKA activity. This weak activation of NKA by low concentration of ouabain may soon disappear after ouabain completely binds to the drug binding pocket, including the binding sites located in the H3-H4 and H5-H6 hairpins of the enzyme. Under our experimental conditions reported in Fig. 3, no significant ouabain stimulation was detected when NKA was incubated with ouabain at 4 °C for 60 min prior to the enzyme activity assay, presumably due to a longer incubation time allowing ouabain to interact with all its binding sites on NKA.

Natural property of NKA

In our previous work, we have identified an activation site of NKA that resides in the H7-H8 domain of the α -subunit of the enzyme and is not a digitalis interaction site [28]. Activator SSA412 can markedly enhance NKA activity by binding to this activation site of the enzyme [28]. This similar phenomenon of accelerated catalytic activity of NKA is detected again when different activator mAb SSA78 or pSSA78 interact with the H1-H2 domain of the enzyme (Figs. 2–4). These findings strongly implicate that the ability to speed up the rate of catalytic function of NKA may be a natural property of the enzyme that can be initiated by the interaction at the selective sites, including the H1-H2 domain of the enzyme. Fig. 4 shows that binding of mAb SSA78 or pSSA78 to the H1-H2 domain of SHHF rat NKA significantly increased enzyme activity,

demonstrating that this latent functional activity not only resides in healthy animal models (Fig. 2A and B), but is also retained within the H1–H2 domain of NKA in heart failure animal model (Fig. 4).

In summary, our experimental results provide direct evidence to demonstrate that the H1–H2 domain of the α -subunit of NKA is capable of regulating enzyme function in two distinct ways for both ouabain-sensitive and -resistant forms of NKA: it increases NKA activity during its interaction with an activator; it also participates in the mechanism of digitalis or ouabain-induced NKA inhibition. Understanding the dual activity of the H1–H2 domain of the enzyme not only helps to deeper understand the biological processes mediated by NKA, but also may lead to the development of new drugs and therapeutic strategies to improve the treatment of heart disease.

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